Acidified Sodium Chlorite as an Alternative to Chlorine for Elimination of *Salmonella* on Alfalfa Seeds

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ABSTRACT: The health and environmental hazard associated with the use of chlorine for food processing has been documented previously. This study was conducted to determine if acidified sodium chlorite (ASC) could be used to replace calcium hypochlorite ($Ca[OCl]_2$) for disinfection of alfalfa seeds. Contaminated seeds containing approximately 1.5 x 10^7 CFU/g of *Salmonella* were treated with ASC or $Ca(OCl)_2$ at different concentrations and for different periods of time. Results showed that the efficacy of ASC and $Ca(OCl)_2$ for elimination of *Salmonella* on contaminated seeds could be improved greatly by extending the treatment time from the traditional 15 to 45 min. Treatment of seeds with 800 ppm of ASC for 45 min reduced the number of *Salmonella* by 3.9 log units, approximately 1.2 log units higher than that treated with 20000 ppm of $Ca(OCl)_2$. Treatment of seeds with a lower concentration (100 to 400 ppm) of ASC for 45 min reduced the number of *Salmonella* by 1.3 to 2.2 log units. Soaking alfalfa seeds in 800 ppm of ASC for 45 min did not affect seed germination. However, soaking seeds in 20000 ppm of $Ca(OCl)_2$ for 45 min reduced seed germination by 20%. Unlike $Ca(OCl)_2$, antimicrobial efficiency of ASC was not affected by pre-exposure to alfalfa seeds. Data presented also showed that *Salmonella* on newly inoculated seeds that had been stored at 4 °C for less than 7 d were more sensitive to sanitizer treatment than those on seeds that had been stored for 4 wk or longer.

Keywords: acidified sodium chlorite, alfalfa seed, chlorine, disinfection, *Salmonella*

Introduction

Consumption of tainted alfalfa sprouts has been implicated in more than 27 foodborne illness outbreaks during the last 2 decades (IFSN 2005). The sources of pathogens associated with these outbreaks (mainly *Salmonella enterica* and *Escherichia coli* O157:H7) were believed to originate from seeds used in sprouting (FDA 1999). Extensive testing was conducted in mid-1990s to evaluate the potential of using various chemicals for disinfection of alfalfa seeds before sprouting (Jaquette and others 1996; Beuchat 1997). Soaking contaminated seeds in a high concentration of calcium hypochlorite ($Ca[OCl]_2$), sodium hypochlorite (NaOCl), or hydrogen peroxide solution was found effective in reducing the number of *Salmonella* (Weissinger and Beuchat 2000) and *E. coli* O157:H7 (Taormina and Beuchat 1999a, 1999b). Based on these studies, treating seeds with 20000 ppm of $Ca(OCl)_2$ or NaOCl for 10 to 20 min before sprouting has been recommended by the FDA (1999) and adopted by the sprout industry (ISGA 2000) to minimize the risk of sprouts serving as a vehicle for foodborne illness.

Chlorine has been used for many years to treat drinking water and to sanitize food processing equipments and surfaces in processing environments (Wei and others 1985; Beuchat 1998). In spite of its popularity as a disinfecting and bleaching agent, there is an increasing concern about the health hazard and environmental impact associated with the use of chlorine. A number of reports (for example, Morris and others 1992) have shown that chlorine, when making contact with organic matter, can rapidly form carcinogenic by-products. In addition, the antimicrobial efficiency of chlorine is greatly affected by pH of the treatment solution and can be rapidly diminished by making contact with organic matters (Wei and others 1985). Because of the drawback associated with the use of chlorine as mentioned, it is desirable to identify a safer and more effective sanitizer for disinfection of alfalfa seeds destined for sprout production.

Acidified sodium chlorite (ASC) is a broad-spectrum antibiotic (Castillo and others 1999; Hajmeer and others 2004), which can be easily prepared by mixing sodium chlorite ($NaClO_2$) with a "generally recognized as safe" organic acid such as citric acid in an aqueous solution. ASC has been approved by the FDA as a food additive or an antimicrobial treatment for poultry, meat, seafood, and raw agricultural commodities (OFR 2000). A number of studies have demonstrated the potential of using ASC for disinfection of sprouting seeds (Taormina and Beuchat 1999a; Weissinger and Beuchat 2000), cabbage (Inatsu and others 2005), carrot (Gonzalez and others 2004; Cruz and others 2006), and cantaloupe (Park and Beuchat 1999; Lukasik and others 2003). The objective of this study was to: (1) compare the efficacy of ASC and $Ca(OCl)_2$ for disinfection of *Salmonella*-contaminated alfalfa seeds by extending the treatment time and changing the sanitizer concentration, (2) investigate the survival and response to sanitizer treatment of *Salmonella* on dry inoculated seeds stored at 4 °C for different periods of time, and (3) determine the antimicrobial efficacy of ASC and $Ca(OCl)_2$ as affected by pre-exposure of sanitizers to different amounts of alfalfa seeds.

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Materials and Methods

Pathogens and alfalfa seeds

The 4 strains of *Salmonella,* including *Salmonella* Anatum F43178, *Salmonella* Infantis F4319, *Salmonella* Stanley H0558, and *Salmonella* Newport H1275, used in the study were provided by Dr. Patricia Griffin (Center for Disease Control and Prevention, Atlanta, Ga., U.S.A.). All 4 strains have been implicated in sprout-related outbreaks and have been used as testing organisms in earlier investigations (for example, Fett 2002). Antibiotic-resistant derivative of each strain displaying dual resistance to 100 μg/mL of nalidixic acid and 500 μg/mL of streptomycin was isolated via spontaneous mutation and used in this study to facilitate the recovery of inoculated *Salmonella* from contaminated seeds. Noncontaminated alfalfa seeds used in the study were purchased from Caudill Seed Co. Inc. (Louisville, Ky., U.S.A.).

Seed inoculation

Each *Salmonella* strain was grown in 50 mL of tryptic soy broth (TSB) containing 100 μg/mL of nalidixic acid and 500 μg/mL of streptomycin. After incubation at 37 °C for 18 h, cultures of all 4 strains were mixed and centrifuged at 8000 *g* for 5 min to collect the cells. The cell pellet was washed once and resuspended in phosphate buffered saline (PBS; 75 μM, pH 7.1; Gibco/Invitrogen Inc., Carlsbad, Calif., U.S.A.) to make an initial cell density (OD_{600}) of approximately 1, which was equivalent to approximately 2×10^9 CFU/mL. Fifty milliliters of the inoculum cocktail containing a mixture of the 4 *Salmonella* strains were added to each stomacher bag containing 100 g of seeds and the bag was massaged by hand for 1 min. After removing excess inoculum, inoculated seeds were transferred to mesh liners and allowed to dry at room temperature for 48 h in a biosafety cabinet. Dry inoculated seeds were then transferred to stomacher bags and stored at 4 °C until use. The initial concentration of *Salmonella* on dry inoculated seeds was determined to be approximately 6×10^8 CFU/g before storage. Preparation of contaminated seeds containing a 10-fold decreasing number of *Salmonella* (for example, 6×10^7 CFU/g) was made by mixing 900 g of noncontaminated seeds with 100 g of contaminated seeds containing 6×10^8 CFU/g of *Salmonella.* An aliquot of 5 g of inoculated seeds was sampled periodically to determine the effect of storage time on survival and response of surviving *Salmonella* to sanitizer treatment.

Enumeration of *Salmonella* and native bacteria on alfalfa seeds

For enumeration of *Salmonella,* 50 mL of PBS was added to a stomacher bag containing 5 g of testing seeds and the bag containing seeds and PBS was then pummeled at high speed for 2 min using a laboratory Stomacher (Seward Inc., London, U.K.). Appropriately diluted seed homogenates were spread plated onto tryptic soy agar (TSA) supplemented with 100 μg/mL of nalidixic acid and 500 μg/mL of streptomycin (designated TSN hereafter). For enumeration of native bacteria associated with noncontaminated seeds, the same procedure was used except that diluted seed homogenates were spread plated onto TSA instead of TSN.

Preparation of sanitizer solutions

Two sanitizer solutions containing either ASC or $Ca(OCl)_2$ at an initial concentration of 800 ppm and 20000 ppm, respectively, were prepared and tested in the study. ASC solution commercially marketed as SANOVA® by Ecolab (St. Paul, Minn., U.S.A.) was prepared by mixing a given volume of 25% sodium chlorite ($NaClO_2$) solution and a given volume of 50% citric acid solution in water as recommended by the manufacturer to make an initial concentration of 800 ppm. $Ca(OCl)_2$ solution containing 20000 ppm of free chlorine was prepared by dissolving 3.2 g of $Ca(OCl)_2$ in 100 mL of distilled water with vigorous stirring for 10 min. Less concentrated ASC or $Ca(OCl)_2$ solutions were prepared by making a series of 2-fold dilutions using sterile water. Actual concentration of chlorous acid ($HClO_2$) in ASC solution was determined using the spectrophotometric or titration method previously described (Kumar and others 2006). Free chlorine content in $Ca(OCl)_2$ solution was determined using a chlorine detection kit (Hatch Co., Ames, Iowa, U.S.A.).

Seed treatments

Treatment time. Dry inoculated seeds (5 g) that had been stored at 4 °C for 4 to 6 wk and containing approximately 1.5×10^7 CFU/g of *Salmonella* were soaked without agitation for 15, 30, or 45 min in 25 mL of a sanitizer solution containing either 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$. Dry inoculated seeds were also soaked in water for 45 min to determine the initial concentration of *Salmonella* on seeds. After soaking in a given sanitizer solution, seeds were rinsed twice with 30 mL of 0.1% of sodium thiosulfate (Kemp and Schneider 2000) or Dey/Engley (D/E) broth (Difco/BD Diagnostic Systems, Sparks, Md., U.S.A.) to neutralize the residual activity of ASC and $Ca(OCl)_2$, respectively. Treated seed samples were then transferred to a stomacher bag containing 50 mL of PBS and pummeled at high speed for 2 min using a laboratory Stomacher. Appropriately diluted homogenates were then spread plated onto TSN to enumerate the number of surviving *Salmonella* on treated seeds.

Sanitizer concentration. Dry inoculated seeds (5 g) that had been stored at 4 °C for 4 to 6 wk and containing approximately 1.5×10^7 CFU/g of *Salmonella* were soaked for 45 min in 25 mL of a sanitizer solution containing 800, 400, 200, or 100 ppm of ASC or 20000, 10000, 5000, or 2500 ppm of $Ca(OCl)_2$. After soaking, treated seeds were rinsed twice with 30 mL of 0.1% of sodium thiosulfate or D/E broth to neutralize the residual sanitizer activity. The number of *Salmonella* remaining on treated seeds was then determined by the procedure as described previously.

Seed storage time. Dry inoculated seeds were removed after storage at 4 °C for different periods of time (that is, 7 d, 4 wk, 4 mo, and 8 mo) to determine the effect of storage time on the survival of *Salmonella* on inoculated seeds. An aliquot (5 g) of seed was also removed after storage for a given period of time and treated with 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$ for 45 min to determine the effect of storage time on the response of surviving *Salmonella* to sanitizer treatment. Noncontaminated seeds that had been stored at 4 °C for over 6 mo were also treated with 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$ for 45 min to determine the efficacy of either sanitizer for elimination of native bacteria associated with alfalfa seeds.

Preparation of seed pretreated sanitizers

To determine if pre-exposure of sanitizer solutions to organic matters such as fresh produce might affect their antimicrobial efficiency, a series of ASC or $Ca(OCl)_2$ solutions were prepared and pre-exposed to different amounts of noncontaminated seeds. Fifty milliliters of ASC (800 ppm) or $Ca(OCl)_2$ (20000 ppm) were mixed, respectively, with 10, 20, 30, or 50 g of noncontaminated seeds and then incubated at room temperature for 15 min. Seed pretreated sanitizer solution were then removed and used to disinfect contaminated seeds that had been stored at 4 °C for 4 to 6 wk and containing approximately 1.5×10^7 CFU/g of *Salmonella* for 45 min.

The number of *Salmonella* remaining on disinfected seeds was determined using the procedure described previously. Antimicrobial efficiency of each seed pretreated sanitizer, as indicated by log microbial reduction, was calculated using the formula: [Number of *Salmonella* (log CFU/g) remaining on inoculated after treatment with water] – [Number of *Salmonella* (log CFU/g) remaining on inoculated seeds after treatment with a seed pretreated sanitizer].

Analysis of seed germination and sprout quality

Noncontaminated seeds (5 g) were soaked in 25 mL of a sanitizer solution in a glass jar containing either 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$ for 15, 30, and 45 min. Seeds soaked in sterile water were used as the control. Following the sanitizer treatment, seeds were rinsed twice with 50 mL of sterile water. One hundred seeds from each treatment were then transferred to a Petri dish containing a wet filter paper and allowed to germinate at room temperature for 2 d. Seeds with germinating roots visible with naked eyes were considered germinated. The tests were repeated 3 times.

Following sanitizer treatment, noncontaminated seeds (5 g) were sprouted in a glass jar at room temperature (approximately 20 °C) for 6 d (Rajkowski and Thayer 2001). Irrigation was carried out daily by adding 1 mL of sterile water into each glass jar during sprouting. Mature sprout in the glass jar was then transferred and stored at 10 °C for up to 5 d. The quality of sprout was scored at day 1, 3, and 5 on an arbitrary scale from 5 to 1 as previously described (Taormina and Beuchat 1999b): 5 = excellent; 4 = good; 3 = average; 2 = poor; and 1 = inedible.

Statistical analysis

The changes in the populations of *Salmonella* on seeds before and after treatments with different concentrations of ASC or $Ca(OCl)_2$ and for different periods of time were analyzed by performing analysis of variance (ANOVA) to determine the effect of treatment time and treatment concentration. Responses of *Salmonella* present on seeds to sanitizer treatments were also analyzed by variance analysis. Difference between treatments were performed using the Bonferroni least significance difference (LSD) mean separation procedure (Miller 1981) at the $P = 0.05$ level.

Results and Discussion

Improving the efficacy of ASC for elimination of *Salmonella* on alfalfa seeds by extending the treatment time

It has been documented previously that disinfection of alfalfa seeds with 20000 ppm of $Ca(OCl)_2$ or other sanitizers for 15 to 20 min can not completely eliminate or reduce the pathogen contamination to an acceptable level (FDA 1999; Fett 2002). To determine if the efficacy of ASC or $Ca(OCl)_2$ for disinfection of alfalfa seeds could be improved by extending the treatment time, contaminated seeds that had been stored at 4 °C for 4 to 6 wk and containing approximately 1.5×10^7 CFU/g of *Salmonella* were soaked in 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$ for 15, 30, or 45 min, respectively. The number of *Salmonella* remaining on seeds before and after soaking was determined. Results (Table 1) showed that the efficacy of either sanitizer for disinfection of seeds was not significantly ($P < 0.05$) affected by extending the treatment time from 15 to 30 min. However, a significant ($P < 0.05$) increase in the reduction of *Salmonella* was observed if treatment time was further increased from 30 to 45 min. Results showed that the number of *Salmonella* remaining on seeds that had been treated with ASC for 30 and 45 min was reduced by 2.4 and 4 log units, respectively. The efficacy of $Ca(OCl)_2$ for disinfection of contaminated seeds was also significantly ($P < 0.05$) improved by extending the treatment time. Results (Table 1) showed that reduction in the number of *Salmonella* increased from 1.7 to 2.8 log units if treatment of seeds with $Ca(OCl)_2$ was extended from 30 to 45 min.

A number of studies reported before (see review by Montville and Schaffner 2004) have shown that treatment of contaminated seeds with sanitizers for 3 to 15 min exerts no significant effect on reduction of *Salmonella* or *E. coli* O157:H7. Results presented here also indicated that the efficacy of ASC or $Ca(OCl)_2$ for elimination of *Salmonella* on dry inoculated seeds could not be improved by extending the treatment time from 15 to 30 min. However, a significant increase in the reduction of *Salmonella* was observed if treatment time was further extended from 30 to 45 min. Data presented (Table 1) also showed that ASC appeared to be more effective than $Ca(OCl)_2$ for seed disinfection. For example, treatment of seeds with 800 ppm of ASC for 45 min resulted in the reduction of *Salmonella* by 3.9 log units, approximately 1.2 log units higher than that treated with 20000 ppm of $Ca(OCl)_2$.

It should be noted, however, that extending the treatment time to improve the efficiency of ASC and $Ca(OCl)_2$ can be used to disinfect sprouting seeds but not for fresh produce with easily damaged surfaces. Sprouting seeds are anatomically protected by a hardy seed coat in nature and are more resistant to the treatment by a chemical sanitizer at an elevated concentration and for a longer period of time. By comparison, leafy vegetables and soft fruits are in general more susceptible to the toxic effect of sanitizer treatments and can sustain the treatment by a sanitizer at a relatively lower concentration (for example, 50 to 200 ppm of chlorine) and for a shorter period of time (for example, 3 to 5 min) (Beuchat 1998).

Efficacy of ASC and $Ca(OCl)_2$ for elimination of *Salmonella* on alfalfa seeds as affected by sanitizer concentration

Treatment of contaminated seeds in 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$ for 45 min had been shown to produce the

Table 1 – Efficacy of ASC and $Ca(OCl)_2$ for elimination of *Salmonella* on alfalfa seeds as affected by the treatment time.[a]

	Contaminated seeds treated with:			
	ASC (800 ppm)		$Ca(OCl)_2$ (20000 ppm)	
Treatment time (min)	*Salmonella* population recovered (log CFU/g)	Log (CFU/g) reduction	*Salmonella* population recovered (log CFU/g)	Log (CFU/g) reduction
0 (water only)	7.45 ± 0.21 A[b]	Control	7.38 ± 0.13 A	Control
15	5.32 ± 0.16 B	2.1	5.67 ± 0.32 B	1.7
30	5.07 ± 0.32 B	2.4	5.35 ± 0.24 B	2.0
45	3.31 ± 0.25 C	4.0	4.68 ± 0.41 C	2.8

[a]Treatment was conducted at room temperature (approximately 20 °C), without agitation, and for a given period of time as specified above.
[b]Each value represents the mean of 6 determinants ($n = 6$) from 3 experiments and 2 duplicates ± standard deviation. Within a column, the numbers not followed by the same letter are significantly different ($P < 0.05$) by the Bonferroni least significant difference (LSD) separation technique (Miller 1981).

greatest reduction in the number of *Salmonella* as presented previously. To determine if the same degree of disinfection efficacy could be achieved using a lower concentration of ASC or $Ca(OCl)_2$, dry inoculated seeds were soaked in a series of 2-fold diluted sanitizer solutions for 45 min. The number of *Salmonella* remaining on seeds before and after treatment of seeds with either sanitizer at different concentrations was determined and summarized as shown in Table 2. A positive correlation was observed between the number of *Salmonella* reduced and the concentration of sanitizer tested if the treatment time was kept at 45 min. However, if treatment time was reduced to 10 min, as reported earlier by Weissinger and Beuchat (2000), no significant ($P < 0.05$) difference in the number of surviving *Salmonella* was found on seeds that had been treated with 500 or 1200 ppm of ASC. Similarly, if treatment time was further reduced to 0.5 to 2 min, as reported earlier by Taormina and Beuchat (1999b), there was no significant ($P < 0.05$) difference in the number of surviving *E. coli* O157:H7 remaining on seeds that had been treated with 500 or 1200 ppm of ASC. These results show that optimum disinfection efficiency is achieved after 45 min of sanitizer treatment.

Results (Table 2) showed that treatment of contaminated seeds with a lower concentration of sanitizer (for example, 200 ppm ASC or 2500 ppm $Ca[OCl]_2$) was very ineffective in reducing the pathogens. Treatment of seeds with either sanitizer at indicated concentration for 45 min only reduced the number of *Salmonella* by 1.3 and 0.8 log units, respectively. It has been reported before (Kumar and others 2006) that soaking mung bean seeds containing 10^3 to 10^4 CFU/g of *Salmonella* in 200 ppm of an oxychloro-based sensitizer (commercially known as Germin-8-or) for 24 h can reduce *Salmonella* to an undetectable level. In this study, soaking dry inoculated seeds containing 1.5×10^7 CFU/g of *Salmonella* in 100 to 200 ppm of ASC for 14 h only reduced *Salmonella* by 1 to 2 log units. This study (data not shown) showed that treatment of contaminated seeds containing 10^3 to 10^4 CFU/g of *Salmonella* with 100 to 200 ppm of ASC for 14 h was unable to eliminate *Salmonella* to an undetectable level.

Antimicrobial efficiency of ASC not affected by pre-exposure to alfalfa seeds

To investigate if the disinfection efficiency of ASC and $Ca(OCl)_2$ might be affected by pre-exposure to organic matters, a series of seed pretreated ASC and $Ca(OCl)_2$ solutions were used to disinfect contaminated seeds. Seed pretreated ASC and $Ca(OCl)_2$ solutions were prepared by preexposure of either sanitizer to different amounts of noncontaminated seeds as described in Materials and Methods. The number of *Salmonella* remaining on seeds that had been disinfected with a seed pretreated sanitizer was determined. Results (Figure 1) showed that the disinfection efficiency of seed pretreated ASC solutions was not significantly ($P < 0.05$) different from the control solution not exposed to noncontaminated seeds. The concentration of chlorate in seed pretreated ASC solutions fell within the range of 740 to 800 ppm, which was close to that detected in control solution not exposed to noncontaminated seeds.

By contrast, the disinfection efficiency of $Ca(OCl)_2$ solutions was greatly affected by pre-exposure of $Ca(OCl)_2$ solutions to different amounts of noncontaminated seeds. For example, treatment of contaminated seeds with a seed pretreated $Ca(OCl)_2$ solution, which was prepared by submerging 10 g of noncontaminated seeds in 50 mL of ASC solution, reduced the number of *Salmonella* by 2.4 log units. By comparison, treatment of contaminated seeds with a seed pretreated $Ca(OCl)_2$ solution, which was prepared by submerging 50 g of noncontaminated seeds in 50 mL, reduced the number of *Salmonella* by only 0.3 log unit. Pre-exposure of $Ca(OCl)_2$ solution to an increasing amount of noncontaminated seeds can result in the reduction in disinfection efficiency. Free chlorine content in $Ca(OCl)_2$ solutions pre-exposed to 10, 20, 30,

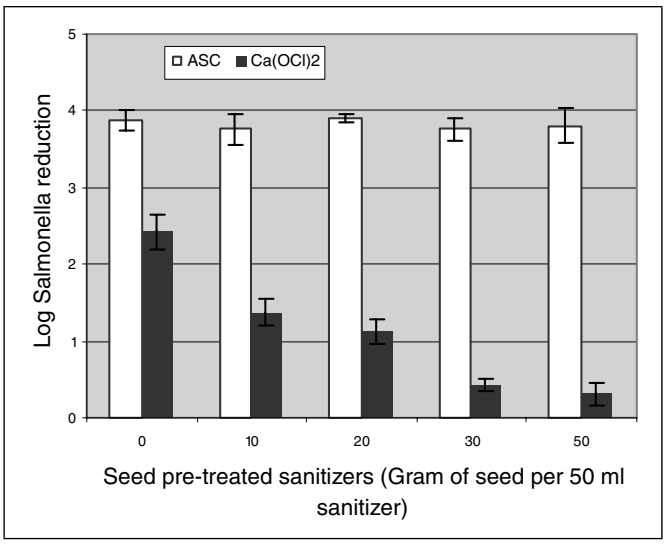

Figure 1—Comparison of the efficacy of ASC (□) and $Ca(OCl)_2$ (■) for elimination of *Salmonella* on contaminated seeds as affected by pre-exposure of sanitizers to different amounts of noncontaminated seeds. Seed pretreated sanitizers were prepared by mixing 50 mL of 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$, respectively, with 0, 10, 20, 30, or 50 g of noncontaminated seeds and shown as 0, 10, 20, 30, and 50 in x-axis. Seed pretreated sanitizers were used to disinfect contaminated seeds for 45 min and the reduction in *Salmonella* was determined and shown as log *Salmonella* reduction in y-axis.

Table 2—Efficacy of ASC and $Ca(OCl)_2$ for elimination of *Salmonella* on alfalfa seeds as affected by sanitizer concentration.[a]

	Contaminated seeds treated for 45 min with:				
	ASC		$Ca(OCl)_2$		
Conc. (ppm)	*Salmonella* population recovered (log CFU/g)	Log CFU/g reduction	Conc. (ppm)	*Salmonella* population recovered (log CFU/g)	Log CFU/g reduction
0 (water)	7.51 ± 0.24 A[b]	Control	0	7.38 ± 0.21 A	Control
100	6.26 ± 0.31 B	1.3	2500	6.57 ± 0.17 B	0.8
200	6.16 ± 0.18 B	1.4	5000	6.45 ± 0.28 B	1.0
400	5.34 ± 0.16 C	2.2	10000	5.41 ± 0.10 C	2.0
800	3.59 ± 0.25 D	3.9	20000	4.70 ± 0.22 D	2.7

[a]Treatment was conducted at room temperature, without agitation, and for 45 min in a sanitizer solution at a given concentration as specified above.
[b]See legend of Table 1.

or 50 g also showed a sharp decline from the initial 20000 ppm to approximately 8000, 4000, 1000, and 200 ppm, respectively.

Previously, it has been shown (Taormina and Beuchat 1999a) that submerging 10 g of alfalfa seeds in 50 mL of chlorinated water for 15 min can reduce the content of free chlorine in the solution by 20%. Fett (2002) also showed that the reduction in free chlorine content was dependent upon the ratio of seed-to-sanitizer volume tested. The lowest level of free chlorine was found in a solution prepared by submerging 50 g of noncontaminated seeds in 50 mL of $Ca(OCl)_2$ solution. To maintain an active level of free chlorine and to maximize the antimicrobial activity of treatment solution, the volume of chlorinated water recommended for seed disinfection should be at least 5 times of seed weight to minimize the reduction in the level of free chlorine after making contact with organic matters (ISGA 2000). Since antimicrobial activity of ASC was not affected by pre-exposure to alfalfa seeds, the volume of ASC required for seed disinfection could be reduced to a volume less than 5 times of the seed weight previously recommended for $Ca(OCl)_2$ (FDA 1999).

Survival and response to sanitizer treatments of *Salmonella* on dry inoculated seeds

The fate and response to ASC or $Ca(OCl)_2$ treatment of *Salmonella* on dry inoculated seeds that had been stored at 4 °C for different periods of time was investigated. The efficacy of either sanitizer for elimination of native bacteria associated with alfalfa seeds was also determined. Results (Table 3) showed that the population of *Salmonella* on dry inoculated seeds declined (>0.6 log unit) significantly ($P < 0.05$) during the first 7 d of storage. The population of *Salmonella*, however, showed no significant change (<0.2 log unit) on dry inoculated seeds that had been stored at 4 °C for a period ranging from 4 wk to 8 mo. This result was consistent with an earlier report by Beuchat and Scouten (2002), who found that *Salmonella* could survive on dry alfalfa seeds for at least 42 wk. Liao and Fett (2003) also reported the isolation of *Salmonella* from naturally contaminated alfalfa seeds that had been implicated in previous disease outbreaks and had been stored at 4 °C for at least 4 y.

Data presented here (Table 3) also showed that *Salmonella* cells on dry inoculated seeds during the first 7 d of storage were more sensitive to ASC or $Ca(OCl)_2$ than those on inoculated seeds which had been stored for 4 wk or longer. For example, disinfection of dry inoculated seeds that had been stored for less than 7 d with 800 ppm of ASC for 45 min could reduce the number of *Salmonella* by 4.2 to 4.4 log units. By comparison, disinfection of dry inoculated seeds that had been stored for 4 wk to 8 mo with 800 ppm of ASC reduced the number of *Salmonella* by only 3.5 to 3.6 log units. *Salmonella* on dry inoculated seeds that had been stored at 4 °C for different periods of time also responded differently to treatment with 20000 ppm of $Ca(OCl)_2$. After treatment, the number of *Salmonella* remaining on seeds that had been stored for less than 7 d was reduced by 3.3 to 3.5 log units. Whereas after treatment, the number of *Salmonella* remaining on seeds that had been stored for 4 wk to 8 mo was reduced by only 1.9 to 2.1 log units.

Native bacteria present on alfalfa seeds also showed a higher level of resistance to sanitizer treatment than *Salmonella* cells present on freshly inoculated seeds that had been stored for less than 7 d. After soaking noninoculated seeds in 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$ for 45 min, the population of native bacteria on alfalfa seeds that had been stored at 4 °C for at least 6 mo was reduced by only 1.5 and 1.8 log units, respectively. Together, these results showed that prolonged storage of dry alfalfa seeds in the cold significantly increased the resistance of native bacteria and *Salmonella* to sanitizer treatment.

Experimentally inoculated seeds after storage in the laboratory for different periods of time had been used to evaluate the efficacy of various sanitizers for elimination of pathogens on alfalfa seeds. For example, inoculated seeds that had been stored for less than 5 d or 6 wk were used in the studies reported by Kumar and others (2006) and by Weissinger and Beuchat (2000), respectively. Data presented here show that *Salmonella* on dry inoculated seeds after storage at refrigeration temperature for different periods of time responded differently to sanitizer treatment. High variability in disinfection efficacy of various sanitizers as reported earlier (see review by Montville and Schaffner 2004) is possibly in part due to the difference in the storage time of contaminated seeds used in the testing. It is important to indicate the storage condition of inoculated seeds used in the testing when reporting the efficacy of sanitizer for elimination of pathogen contamination.

Table 3—Survival and response to ASC and $Ca(OCl)_2$ treatment of *Salmonella* on dry inoculated seeds after storage at 4 °C for different periods of time.

	Populations (log CFU/g) of native bacteria or *Salmonella* remaining on dry inoculated seeds after treatment with water or sanitizer for 45 min		
Targeted bacteria on seed samples	**Water**	**ASC**	**$Ca(OCl)_2$**
Native bacteria on noninoculated seeds	5.37 ± 0.18	3.53 ± 0.31 (1.84)[a]	3.92 ± 0.23 (1.45)
Salmonella on dry inoculated seeds after storage at 4°C for:			
0 d	7.72 ± 0.34 A[b]	3.29 ± 0.27 A (4.4)	4.21 ± 0.26 A (3.5)
7 d	7.31 ± 0.07 B	3.11 ± 0.15 A (4.2)	3.99 ± 0.34 A (3.3)
4 wk	7.14 ± 0.28 B	3.58 ± 0.31 B (3.6)	5.10 ± 0.15 B (2.0)
4 mo	6.87 ± 0.41 B	3.35 ± 0.16 B (3.5)	4.76 ± 0.24 B (2.1)
8 mo	7.01 ± 0.32 B	3.44 ± 0.27 B (3.6)	5.13 ± 0.18 B (1.9)

[a]The value within parenthesis represents the log difference in the population of *Salmonella* recovered from seeds that had been treated with water and from seeds that had been treated with either 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$. The disinfection efficiency as indicated by the value within the parenthesis decreases as the resistance of *Salmonella* to the treatment increases.
[b]Each value represents the mean of 2 experiments and 2 duplicates in each experiment ($n = 4$) ± standard deviation. Within a column, the numbers not followed by the same letter are significantly different ($P < 0.05$).

Table 4—Effect of sanitization treatment on seed germination and sprout quality in storage.[a]

Seed treatments	% Germination[a]	Quality index of sprout stored at 10 °C for: 1 d	3 d	6 d
Soak in 800 ppm ASC for:				
15 min	93	4.3[b]	4.2	4.0
30 min	89	3.9	4.0	3.7
45 min	91	3.8	3.8	3.7
Soak in 20000 ppm $Ca(OCl)_2$ for:				
15 min	93	4.2	4.0	4.2
30 min	76	2.3	2.7	2.2
45 min	74	2.8	2.2	2.9
Control (water, 45 min)	94	4.0	3.7	4.0

[a]Noncontaminated seeds were treated with 800 ppm of ASC or 20000 ppm $Ca(OCl)_2$ for 15, 30, or 45 min and then subject to sprouting for 6 d. Mature sprouts in the glass jars were stored at 10 °C and sprout quality was determined at day 1, 3, and 6.
[b] Sprout quality index was ranked on a 5 to 1 scale: 5 = excellent (as compared to water control); 4 = good; 3 = average; 2 = poor; and 1 = inedible, as previously defined (Taormina and Beuchat 1999b). Each value represents the mean of 2 experiments and 2 duplicates in each experiment.

Effect of sanitizer treatment on seed germination and sprout quality

Noncontaminated seeds were treated with 800 ppm of ASC or 20000 ppm of $Ca(OCl)_2$ for 15, 30, and 45 min. The germination rate of treated seeds and the quality of produced sprout in storage were determined and summarized as shown in Table 4. Data presented show that treatment of seeds with 800 ppm of ASC for up to 45 min had very little or no effect on seed germination and on sprout quality during storage at 10 °C for 6 d. However, treatment of seeds with 20000 ppm of $Ca(OCl)_2$ for 30 or 45 min reduced seed germination by more than 20% and greatly affected the quality of sprouts during storage. It has been found inappropriate to treat seeds with 20000 ppm of $Ca(OCl)_2$ for longer than 30 min (Weissinger and Beuchat 2000; Fett 2002). However, treatment of seeds with 800 ppm of ASC for 45 min did not show any adverse effect on seed germination and sprout quality. As mentioned previously, increasing the exposure time and sanitizer concentration is suitable for treatment of alfalfa seeds but not for leafy green or salad vegetables with easily damaged surfaces.

Conclusions

Data presented here demonstrated the potential of using ASC to replace $Ca(OCl)_2$ for disinfection of alfalfa seeds. Soaking contaminated seeds in 800 ppm of ASC for 45 min could reduce the number of *Salmonella* by 3 to 4 log units. By comparison, soaking contaminated seeds in 20000 ppm of $Ca(OCl)_2$ for 45 min only reduced the number of *Salmonella* by 2 to 3 log units. Unlike $Ca(OCl)_2$, antimicrobial efficiency of ASC solution was not affected by pre-exposure to organic materials including noncontaminated seeds. A smaller volume of ASC than that required for $Ca(OCl)_2$ may be used for seed treatment. Moreover, treatment of seeds with 800 ppm of ASC for 45 min did not affect seed germination and sprout quality. This study also showed that *Salmonella* on inoculated seeds that had been stored at 4 °C for over 4 mo were more resistant to sanitizer treatment than *Salmonella* on newly inoculated seeds that had been stored for less than 7 d. It is important to specify the storage condition of contaminated seeds used in the study when reporting the efficacy of sanitizer treatment for elimination of pathogens on alfalfa seeds.

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